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Development of a semi-selective medium and an immunofluorescence colony-staining procedure for the detection of Clavibacter michiganensis subsp. sepedonicus in cattle manure slurry

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Abstract

Various compounds and basal media were tested for their suitability to create a semi-selective medium for isolation of *Clavibacter michiganensis* subsp. *sepedonicus* (Cms) from cattle manure slurry containing c. 10^8 colony forming units (cfu) per ml.

Plating efficiency of Cms in yeast glucose mineral medium (YGM) was 104% compared with yeast peptone glucose medium. Nalidixic acid, polymyxin B sulphate and the experimental disinfectant S-0208 inhibited colony growth of cattle slurry bacteria as compared with Cms in YGM. The optimal concentration of these inhibitors in combination was determined by modified agar diffusion tests and by pour plating in 24-well tissue culture plates. The semi-selective medium YGMI consisted of YGM supplemented with nalidixic acid (2 mg/l), polymyxin B sulphate (30 mg/l) and S-0208 (125 mg/l). Plating efficiency varied for Cms between 50.9 and 69.6%, for cattle slurry bacteria between 1.8 and 2.5% and for saprophytes from potato heel end extracts between 11.5 and 27.4%.

Differentiation of Cms colonies from other colonies was based on their small and bluish colony morphology in pour plates and on immunofluorescence colony-staining (IFC). IFC of a pure culture of micro colonies of Cms in YGM was possible after one day incubation (colonies c. 5 cells). Green background fluorescence in the agar gels was prevented by addition of Tween 20 (0.1%) to the washing buffer and the use of 1% agar gels. IFC of macro colonies of Cms in YGMI, visible with 4 x objective magnification, was possible after 4 days. The detection level of the target organism in artificially inoculated cattle slurry in YGMI based on colony morphology varied between 1.4×10^3 and 2.3×10^4 cfu per ml of cattle slurry. Miniaturized plating combined with IFC, using wells in tissue culture plates ($\varnothing$ = 16 mm), proved suitable for detection, but was c. 30 times less sensitive. The recovery of Cms was negatively correlated with the number of saprophytic colonies in the agar plates (R^2 = 0.74).

Additional keywords: antibiotic screening, colony differentiation, cross-reaction, detection level, fluorescent background reduction, micro colony assay, miniaturized agar plating, plating efficiency, potato heel end.

Introduction

Clavibacter michiganensis subsp. *sepedonicus* (Cms), the causal organism of ring rot, is an important pathogen of potatoes in temperate climate regions. Since ring rot has

never been reported in the Netherlands, extensive quarantine measures were taken to prevent its introduction by importing Cms contaminated potatoes. A possible way to introduce Cms is by importing contaminated tubers used for animal nutrition. Cattle or pig manure slurry might become contaminated with Cms and serve as a carrier to bring the pathogen into the agricultural system. No suitable method is yet available to study the survival of Cms in cattle manure slurry.

A standardized test procedure for Cms based on screening of potato heel end extracts with immunofluorescence (IF) and a pathogenicity test on eggplant has been developed by the ad hoc Expert Committee on Bacterial Ring Rot of the European Community. Confirmation of IF-results was considered necessary because cross-reacting organisms are known to occur in potato tuber samples (Miller, 1984; Janse and Van Vaerenbergh, 1987). Isolation procedures in general failed for this purpose and therefore a sensitive but time consuming pathogenicity test is now used for confirmation of IF-positive samples.

For detection of *Erwinia* spp. in cattle slurry, we have compared IF with other methods. Because of the presence of many interfering particles from the cattle slurry in preparations for IF, the detectability of *Erwinia* spp. with IF was very poor e.g. as compared with isolation methods. The best detection of *Erwinia carotovora* subsp. *atroseptica* and of *E. chrysanthemi* was obtained with pour plating of the cattle slurry samples in a semi-selective medium in combination with immunofluorescence colony-staining (IFC) to differentiate between target and saprophyte colonies. In comparison with surface plating, pour plating of the sample allowed a significant increase in the number of colonies per plate. The detection level of this assay in a routine format, using the 16 mm diameter wells in 24-well tissue culture plates instead of 9 cm diameter Petri dishes, was still c. 100 colony forming units (cfu) for both *Erwinia* spp. per ml of undiluted cattle slurry (Van Vuurde and Roozen, 1990).

The objective of this study was to investigate the use of pour plating in combination with IFC for the detection of Cms in cattle slurry. Because of the importance of a sensitive and specific detection method for Cms for e.g. potato indexing, the final procedure was also tested for potato heel end extracts.

Materials and methods

Bacterial strains, cultural and incubation conditions

The following strains of Cms were used: IPO (Research Institute for Plant Protection) no. 498, isolated from potato in the USA, no. 269 and 270, isolated from potato in Sweden. Strains were grown on slope cultures of an agar medium containing per liter: 8 g Lab-Lemco broth (Oxoid CM15), 5 g NaCl, 15 g agar (Oxoid L13) for 72 h at 20 °C. Dilutions of the cultures were prepared in quarter strength Ringer's solution (Becton Dickenson, 1985).

The plating efficiencies of Cms, saprophytes from potato heel ends and from cattle slurry were determined in various media by pour plating. Suspensions containing c. 1000 cfu per ml were mixed through the media at 45 °C in Petri dishes (∅ = 9 cm) or wells of tissue culture plates (∅ = 16 mm). Plate counts were made after 5 days incubation at 20 °C.

Preparation of samples

Cattle slurry. Two cattle slurry samples were obtained from the experimental farm 'De Ossenkampen' in Wageningen. They had a dry matter content of 7.4% and a pH of 7.6. Subsamples were stored at −80 °C in plastic bags containing c. 50 ml of cattle slurry, and were thawed and inoculated with the target organism as described earlier (Van Vuurde and Roozen, 1990). Total counts of bacteria were determined monthly for the stored subsamples by pour plating of sample dilutions in trypticase soy agar (TSA, BBL no. 11043). The samples contained c. 10^8 cfu per ml. Inoculated Cms bacteria were exposed less than 15 min to the cattle slurry before plating.

Potato heel end. Heel end cores from Dutch seed potatoes of the cultivars Baraka and Diamant were removed according to the procedure described by the Commission of the European Communities (1987). The potato cores were macerated and suspended in 50 ml of 0.01 M phosphate buffered saline (PBS, pH 7.4).

Selectivity for Cms of C- and N-sources and inhibitors

Carbon sources, nitrogen sources and inhibitors were tested for their effect on the plating efficiency of Cms and of cattle slurry bacteria. Based on Snieszko and Bonde (1943) and Paquin and Pelletier (1969) the following carbon sources were tested: glucose; dextrose; raffinose; sorbitol; mannitol; sodium gluconate; asparagine; and fructose at a concentration of 2.5 g/l. They were tested in two basal media, the first contained per liter: 5 g yeast extract (Oxoid L21), 10 g bacteriological peptone (Oxoid L37) and 15 g agar and the second basal medium (De Bruyne, 1985) contained per liter: 10 mg yeast extract and 1 g KNO_3 and 15 g agar. Based on Lachance (1962) and Paquin and Lachance (1970) the following nitrogen sources were tested: casein hydrolysate; tryptose; a combination of yeast and peptone; a combination of asparagine, L-methionine, hystidine, leucine, alanine, arginine and proline; and this combination of amino acids plus yeast extract. The final concentration of the N-source was 4.2 g in the basal medium of Lachance (1962). In case of N-sources consisting of various components, the weight of the individual components was equal. The plating efficiencies for Cms and cattle slurry bacteria in these media were compared with yeast extract, peptone, glucose medium (YPG, containing per liter: 5 g yeast extract, 10 g bacteriological peptone, 5 g glucose, 15 g agar).

Potential selective inhibitors were initially screened at one concentration in YPG or yeast glucose mineral medium (YGM, De Boer and Copeman, 1980). The inhibitors were sodium azide (2 mg/l), polymyxin B sulphate (Sigma P- 1004, 7900 USP units per mg, 40 mg/l), LiCl (5 g/l), $Na_2Cr_2O_7$ (0.2 mg/l) and nalidixic acid (Sigma, N-8874, 0.2 mg/l) and the experimental disinfectant S-0208 (Sumuto mo Chem. Co. Osaka Japan, 6.25 mg/l). Nalidixic acid was dissolved in 1 N NaOH and diluted 100 times in demineralized water. S-0208 could not be completely dissolved. The inhibitors for all experiments were freshly prepared, filter sterilized (0.45 μm) and seperately added after autoclaving the agar medium. The volume of each solution of inhibitor was 0.5% of the final volume of the agar medium.

Qualitative screening of inhibitors with an agar diffusion test

This test was used to obtain information on the optimal concentration of nalidixic acid, polymyxin B sulphate, and S-0208 and on possible interaction between these

compounds. Pour plates with Cms (strain 498) or a cattle slurry dilution in YGM were prepared in Petri dishes of 5, 9, 11 and 14 cm diameter. The amount of medium and of the Cms or cattle slurry suspensions were equal per surface area in the plates with different diameters and corresponded with 5 ml medium and 100 µl suspension in a 5 cm Petri dish. Concentration gradients of three inhibitors in one agar plate were created as follow: Three wells (∅ = 5 mm) were cut in the agar at equal distances from each other and from the border of the plate. The wells were filled with 25 µl of a solution of the different inhibitors. After incubation overnight at 4 °C, the plates were further incubated at 20 °C. Plates were screened for areas with a high plating efficiency of Cms and a low plating efficiency of cattle slurry bacteria. Selective activity of an inhibitor was observed by the diffusion gradient that developed between the well and the edge of the plate; interaction between components was determined between the wells.

Polymyxin B sulphate was tested at 80 mg/ml, nalidixic acid at 0.4 mg/ml and S-0208 at 12.5 mg/ml.

Miniaturized quantitative testing for optimum concentration of inhibitors
Pour plates of Cms or cattle slurry suspensions were separately prepared in wells (∅ = 16 mm) of tissue culture plates. YGM (0.5 ml per well) was supplemented with S-0208 at 0, 25 or 125 mg/l, nalidixic acid at 0, 0.4, 2 or 10 mg/l and polymyxin B sulphate at 0, 10, 20, 30 or 40 mg/l. The plating efficiency of Cms and of cattle slurry bacteria for all media with inhibitors at all concentrations was compared with YGM after 5, 9 and 14 days.

Plating efficiency
The plating efficiency of Cms and of cattle slurry bacteria was determined for YPG, YGM, YGM with inhibitors and for semi-selective medium for *C. michiganense* (SCM, Fatmi and Schaad, 1988). SCM was tested with and without potassium tellurite.

The plating efficiency of saprophytes from potato heel ends was determined in YGMI.

Antiserum and conjugate
Anti-Cms serum was prepared against strain 498 according to the method of Vruggink and Maas Geesteranus (1975). The fluorescein isothiocyanate (FITC)- conjugate IPO no. 7649c/5 was prepared according to the method of Allen and Kelman (1977).

Immunofluorescence colony-staining (IFC)
Macro colony (> 0.1 mm) technique. Agar discs (∅ = 16 mm) from pour plates (7.5 ml medium per 9 cm Petri dish) were dried and stained with FITC-conjugated antiserum (1 : 100 diluted in PBS) as described earlier (Van Vuurde and Roozen, 1990). Agar films with colonies from cattle slurry without target bacteria and agar films with colonies of the target bacterium were used as negative and positive controls, respectively.

Micro colony (< c. 0.01 mm) technique. Cms pour plates were prepared similar to those for macro colonies and incubated for 1 or 2 days for growth of Cms micro colonies visible at 400× magnification. After incubation, agar discs (∅ = 8 mm) were

dried on glass slides and stained as described for macro colonies FITC-conjugate (1 : 300 diluted in PBS). Reduction of background staining of IFC preparations of micro colonies was tested at agar concentrations of 0.5, 1.0, 1.5 and 2.0%. Each of these concentrations was incubated with FITC-conjugated serum to which one of the following compounds was added: Tween 20 (0.1%), pre-immune serum (IPO no. 8575a/1, 10%), Evans blue (0.1%) or Merck bromthymol blue (0.1%, Chroma Ges.). Another treatment involved an increase of washings to 6 times 10 min after incubation with the conjugate.

Microscopy
Colonies in pour plates were examined for morphological differences with a Wild M7S stereomicroscope with darkfield illumination at 30× magnification.

Immunofluorescence microscopy
Preparations for IFC- examination were observed with a Leitz Orthoplan microscope with an internal tube magnification factor of 1.25 and equiped with incident blue light and filter system for FITC (Leitz I_2). IFC-stained macro colonies in agar films were examined with a 4× objective (Leitz achromat, NA 0.12) and 4× ocular, micro colonies with a 40× objective (Leitz plan objective, NA 0.65).

Statistical analysis
Analysis of variance and tests for least significant difference were used for comparison of data.

Results

Comparison of C- and N-sources and media
None of the tested carbon or nitrogen sources influenced the plating efficiency of Cms and cattle slurry bacteria in the basal media compared with YPG. In the non-supplemented medium of Lachance, the plating efficiency of Cms was equal with YPG but the colonies remained about 0.75 times smaller in diameter than in YPG. The plating efficiency for Cms and cattle slurry bacteria in YGM was respectively 104% and 109% compared with YPG.

SCM and its modification without potassium tellurite did not give colony growth of Cms within 21 days neither by pour plating nor by surface dilution plating. For SCM the plating efficiency of cattle slurry bacteria was 0.1% and 3.2% for this medium with and without potassium tellurite, respectively.

Selective effect of inhibitors
In the medium with sodium azide no growth occurred at all. Lithium chloride did not influence the plating efficiency of cattle slurry bacteria. No Cms colonies developed in the medium with $Na_2Cr_2O_7$. The results of the various inhibitors giving a high plating efficiency of Cms and suppression of cattle slurry saprophytes are presented in Table 1. Nalidixic acid and polymyxin B sulphate reduced the plating efficiency of cattle slurry bacteria to respectively 21 and 22%, whereas the plating efficiency of Cms was similar to YPG or YGM. In YPG with both nalidixic acid and polymyxin B sulphate the Cms colonies were c. half length and width compared with YPG. The experi-

Table 1. The plating efficiency of *Clavibacter michiganensis* subsp. *sepedonicus* (Cms) strain 498 and indigenous cattle slurry bacteria in YPG or YGM supplemented with selective inhibitors. Plate counts are made after 6 days incubation. Data represent the average of 5 replicates. The average number of cfu in basal medium is considered 100%. Values with dissimilar superscripts (horizontal and vertical comparison) are significantly different at $P = 0.05$.

Inhibitor (concentration: mg/l)	Basal medium	Plating efficiency (%)	
		Cms	cattle slurry bacteria
Nalidixic acid (0.2)	YPG	102[a]	21[b]
Polymyxin B sulphate (40)	YPG	105[a]	22[b]
S-0208 (6.25)	YGM	75[c]	19[b]

mental disinfectant S-0208 added to YGM gave a plating efficiency of 75% for Cms and of 18% for cattle slurry bacteria.

Optimal concentration of inhibitors

An agar diffusion test was used to study the effect of a concentration gradient of the selective inhibitors nalidixic acid, polymyxin B sulphate and S-0208 on growth of Cms and of cattle slurry saprophytes. The wells filled with polymyxin B sulphate were surrounded after 1 day by a slightly milky halo ($r = 1$ cm) where no colonies developed, followed by a zone (r increased from 1 to 2 cm) with a gradient of an increasing number of colonies. Wells with S-0208 or nalidixic acid were surrounded by a zone in which the plating efficiency of cattle slurry bacteria increased from 20% near the well up to 100% at a distance of 2.5 cm from the well. No reduction in plating efficiency of Cms was noticed in these experiments except for the 1 cm halo around the well with polymyxin B sulphate. Combinations of promising concentrations of these inhibitors in YGM were further evaluated for separately pour plated suspensions of cattle slurry and of Cms (Table 2). In general the results confirmed those in Table 1. No inhibitor reduced the plating efficiency of cattle slurry bacteria to less than 10 to 20% without a reduction of the plating efficiency of Cms. Several combinations of concentrations of the three selective inhibitors gave a high plating efficiency of Cms ($> 90\%$) and a low plating efficiency of cattle slurry bacteria ($< 10\%$). In some cases (see Table 2) colony growth from cattle slurry bacteria was temporarily retarded and increased between 5 and 14 days. The YGM supplemented with the inhibitors at concentrations of 2 mg/l nalidixic acid, 30 mg/l polymyxin B sulphate and 125 mg/l S-0208 was chosen for further experiments and referred to as YGMI.

Plating efficiency in semi-selective medium

The plating efficiency in YGMI was compared with YGM by pour plating three Cms strains, saprophytes from heel ends of two potato varieties and from two cattle slurry samples (Table 3). The Cms strains had a plating efficiency between 50.9 and 69.6%. The plating efficiency for the saprophytes from the two cattle slurry samples was 1.8% and 2.5%, and from the potato heel end samples 27.4% for the variety Baraka and 11.5% for Diamant.

Table 2. The plating efficiency of *Clavibacter michiganensis* subsp. *sepedonicus* (Cms) strain 498 and of saprophytic cattle slurry bacteria (sap.) in YGM supplemented with different concentrations of nalidixic acid, polymyxin B sulphate and S-0208. The plating efficiency has been scored after 5 and 14 days of incubation in classes of cfu compared with YGM. Each figure represents the average of 2 replicates after 5 days of incubation.

Nalidixic acid (mg/l)	S-0208 (mg/l)	Polymyxin B sulphate (mg/l)									
		0		10		20		30		40	
		Cms	sap.	Cms	sap.	Cms	sap.	Cms	sap.	Cms	sap.
0	0	4[1]	4	4	3	4	3	3↑	3	3.5↑	2↑
0	25	4	2.5	4	2	4	1.5↑	4	1.5↑	3.5↑	1↑
0	125	4	3	4	2	4	1↑	4	1↑	4	1
0.4	0	4	4	4	3	4	3	4	2.5	2↑	2.5↑
0.4	25	4	3	4	2	4	1.5↑	4	1↑	3↑	1↑
0.4	125	4	3	4	2	4	1↑	4	1	3↑	1
2	0	4	2.5↑[2]	4	2↑	4	2.5↑	4	2↑	2↑	1
2	25	4	2↑	4	2	4	1↑	4	1↑	3↑	1
2	125	4	2↑	4	2	4	1↑	4	1	3↑	1
10	0	2	2↑	4	2	4	1.5↑	4	1	3↑	1
10	25	4	2↑	4	1.5↑	4	1.5↑	4	1	2↑	1
10	125	4	2↑	3.5↑	1.5↑	3	1.5↑	4	1	1.5↑	1

[1] Plating efficiency classes: 1 = 0-10%; 2 = 11-50%; 3 = 51-90%; 4 = 91-100%.
[2] ↑ indicates an increase of cfu between 5 and 14 days.

Table 3. Plate counts in yeast glucose mineral medium (YGM) and in YGM with inhibitors (YGMI) and plating efficiency of YGMI for *Clavibacter michiganensis* subsp. *sepedonicus* (Cms) and for saprophytes from two cattle slurry samples and from samples of potato cultivars Diamant and Baraka. Data represent average and standard deviation of replicates. The average number of cfu in YGM is considered 100%. Values with dissimilar superscripts (horizontal comparison) are significantly different at $P = 0.05$.

Sample	Number of cfu		Plating efficiency YGMI (%)
	YGM	YGMI	
Cms 498	146 ± 18[a]	88 ± 18[b]	60.3
Cms 269	138 ± 34[a]	96 ± 23[a]	69.6
Cms 270	228 ± 23[a]	116 ± 25[b]	50.9
Cattle slurry #1	4577 ± 652[a]	117 ± 13[b]	2.5
Cattle slurry #2	2672 ± 471[a]	48 ± 4[b]	1.8
Potato cv. Diamant	4204 ± 953[a]	484 ± 81[b]	11.5
Potato cv. Baraka	624 ± 109[a]	171 ± 22[b]	27.4

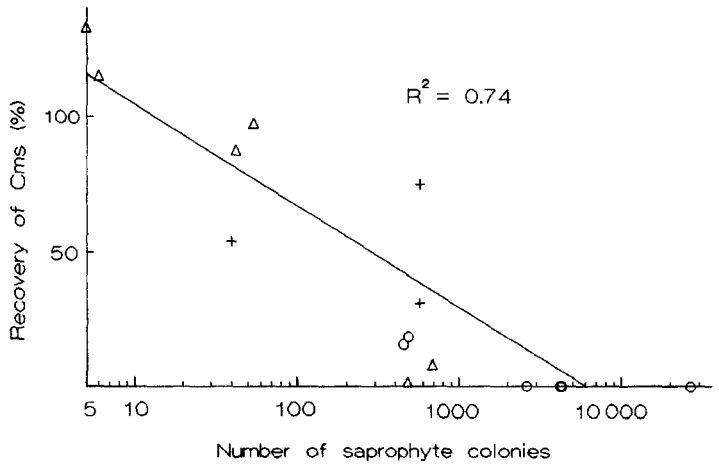

Fig. 1. Relation between the number of saprophyte colonies per Petri dish (∅ = 9 cm) from cattle slurry and the recovery of *Clavibacter michiganensis* subsp. *sepedonicus* in yeast glucose mineral (YGM) medium (o), YGM with nalidixic acid (0.2 mg/l) and polymyxin B sulphate (40 mg/l) (△); YGM with nalidixic acid (2 mg/l), polymyxin B sulphate (30 mg/l) and S-0208 (25 mg/l) (+).

Interference of saprophytes with the plating efficiency of Cms

Colony growth of Cms was generally hampered by saprophyte colonies in the agar pour plates (Fig. 1). A density of about 120 saprophyte colonies in a 9 cm ∅ Petri dish reduced the recovery of Cms to 50% and c. 1200 saprophyte colonies to 10%.

Differentiation between Cms and saprophyte colonies

A. Differentiation by colony morphology. In YGMI pour plates with saprophyte colonies, Cms colonies developed rather specific characteristics between 4 to 6 days: they were much smaller and bluish compared with colonies of saprophytes. Cms in cattle slurry could be detected in YGMI by microscopic examination of the plates for colony morphology. Differentiation was not present when plates were incubated more than 6 days or when Cms was grown in a medium in which no inhibitors were present. In non-inoculated slurry only 0.6% of the colonies from cattle slurry bacteria showed morphological resemblance with Cms colonies in YGMI.

B. Differentiation with IFC. Cms colonies could be stained with FITC-conjugated serum from 1 day incubation onwards. After 1 day, Cms micro colonies consisted of c. 5 cells. Various treatments were tested to reduce the high green fluorescent background that was present when stained micro colonies were observed with a 40 × objective (Table 4). Addition of Evans blue (0.1%) or pre-immune serum (10%) to the conjugated serum did not reduce the background staining. Bromthymol blue (0.1%) turned the colonies red. Six washings after the incubation of the conjugate were not effective, whereas the addition of 0.1% Tween 20 to the dilution buffer of the conjugate and to the washing buffer and the use of 1% agar gels greatly reduced the background fluorescence.

Table 4. Effect of agar concentration and of additives to the FITC-conjugated antiserum against *Clavibacter michiganensis* subsp. *sepedonicus* (Cms) on colony-staining (CS) and staining of the background (BS) of 2 days incubated pour plates of Cms (strain 269). Total magnification 200×, objective 40×.

Agar concentration (%)	Additive									
	None		Evans blue		Bromthymol blue		Tween 20		Pre-immune serum	
	CS	BS	CS	BS	CS	BS	CS	BS	CS	BS
0.5	4[1]	0	4	0	0[2]	0	4	0	4	0
1.0	4	1	4	1	0[2]	0	4	0	4	2
1.5	4	3	4	1	0[2]	1	4	1	4	3
2.0	4	3	1	1	0[2]	1	4	1	4	3

[1] Classes of fluorescence: 0 = no immunofluorescence, 1 = slightly green, 2 = moderately green, 3 = clearly green, 4 = brilliant green.
[2] Colonies were stained slightly red.

Micro colonies, observed with a 40 × objective were brilliantly stained at a 1 : 300 dilution of the conjugate. A dilution of 1 : 100 was needed for a brilliant staining of Cms colonies observed with a 4 × objective, due to the 5.5 × lower numerical aperture.

The number of cross-reacting colonies in non-inoculated slurry was dependent on the medium. In YPG and YGM 0-0.16% of the examined colonies cross-reacted whereas in YGMI and YPG with inhibitors 4-5% cross-reacted. Cross-reacting colonies, however, could be easily differentiated from Cms colonies because the staining of these colonies was fainter and they were bigger than those of Cms. Autofluorescent colonies were not found.

Detection of Cms in cattle slurry

A. Based on colony morphology. At an inoculation level of 1.4×10^4 (strain 498) or 2.3×10^4 (strain 270) cfu of Cms, the number of Cms-type colonies in YGMI was significantly ($P = 0.05$) higher in inoculated than in non-inoculated cattle slurry. The recovery was 39% and 83%, respectively (Table 5).

B. Based on IFC. Cms was detected in YGMI combined with IFC in agar discs of 16 mm diameter in tissue culture plates. This c. 30 times smaller area than Petri dishes (∅ = 9 cm) was used because of its potential for routine detection. At a level of 1.4×10^5 Cms cfu per ml of cattle slurry the number of Cms-type colonies was significantly higher than in non-inoculated slurry. The recovery at this inoculation level was 100% (Table 6). In YGM without the inhibiting substances Cms was not detected. To evaluate morphological detection of Cms colonies, pour plates were indexed for Cms-type colonies and were stained with IFC. All Cms-type colonies (> 100 colonies tested) reacted positive with the anti-Cms conjugated serum.

Table 5. Plate counts and percentage recovery for two strains of *Clavibacter michiganensis* subsp. *sepedonicus* from artificially inoculated cattle slurry by pour plating 100 µl of 100 times diluted sample in yeast glucose mineral medium with inhibitors (YGMI). Data represent average and standard deviation of colonies with Cms-morphology per Petri dish (∅ = 9 cm) of 5 replicates.

Strain 498			Strain 270		
inoculation concentration[1]	positive colonies	recovery[2] (%)	inoculation concentration	positive colonies	recovery (%)
Non-inoculated	1.0 ± 0.7		Non-inoculated	1.0 ± 1.0	
1.4×10^3	1.4 ± 0.9	29	2.3×10^3	3.2 ± 0.8	96
1.4×10^4	6.4 ± 2.6	39	2.3×10^4	20.2 ± 4.4	83
1.4×10^5	92.2 ± 21.4	65	2.3×10^5	166.4 ± 46.3	72
1.4×10^6	754.0 ± 129.9	54	2.3×10^6	1364.0 ± 373.2	59

[1] The concentration was calculated from plate counts from pour plated dilutions of the pure culture in YGM medium (cfu per ml cattle slurry).
[2] The recovery (%) is corrected for positive colonies in non-inoculated cattle slurry: Recovery (%) = (number of positive colonies - number of positive colonies in non-inoculated cattle slurry) × 100% / (number of positive colonies based on 100% recovery).

Table 6. Plate counts and percentage recovery of *Clavibacter michiganensis* subsp. *sepedonicus* (Cms), strain 498 from artificially inoculated cattle slurry by immunofluorescence colony--staining (IFC) in yeast glucose mineral (YGM) medium and YGM with inhibitors (YGMI). Data represent average and standard deviation of positive colonies per well (∅ = 16 mm) in tissue culture plates with 3.1 µl of 100 times diluted cattle slurry of 5 replicates.

Inoculation concentration	YGM		YGMI		
	IFC-positive colonies	recovery[1] (%)	IFC-positive colonies	Fluorescence of colony	recovery (%)
Non-inoculated	0.0 ± 0.0	–	0.2 ± 0.4	Yellow/green[2]	–
1.4×10^3	0.0 ± 0.0	0	0.4 ± 0.5	Yellow/green[2]	0
1.4×10^4	0.0 ± 0.0	0	0.0 ± 0.0		0
1.4×10^5	0.0 ± 0.0	0	5.4 ± 2.1	Green	122
1.4×10^6	0.0 ± 0.0	0	29.0 ± 13.7	Green	66

[1] The concentration was calculated from plate counts from pour plated dilutions of the pure culture in YGM medium (cfu per ml cattle slurry).
[2] Atypical (large) colony size.

Discussion

The general failure to isolate Cms from natural substrates like soil or heel ends of symptomless potato tubers is the result of the strong dominance of naturally occurring saprophytes on the isolation medium (Janse and Van Vaerenbergh, 1987). The compe-

tition for essential nutrients between Cms and fast growing saprophytes seems the most likely explanation for the extensive inhibition of Cms, even on media specially developed for Cms such as YGM. Other factors may be the production of inhibitory compounds, e.g. toxins or pH reducing compounds (Paquin and Pelletier, 1966) by certain saprophytes. Our research strategy for the development of a sensitive method for the detection of Cms followed two major lines. The first series of experiments was made to optimize the composition of the agar medium for pour plating to improve growth of Cms and to reduce growth of saprophytes from cattle slurry. The second series of experiments was made to investigate the use and specificity of IFC for reliable differentiation between the Cms colonies and those of saprophytes from cattle slurry in pour plates.

Selective Cms medium for pour plating

In the literature, several carbon compounds were compared for their stimulative effect on Cms growth (Snieszko and Bonde, 1943; Paquin and Pelletier, 1969). Paquin and Pelletier (1969) found high growth rates of Cms for sucrose and glucose, average growth rates for fructose and galactose and a very poor growth rate for rhamnose. Lachance (1962) and Paquin and Lachance (1970) compared various amino acids for their effect on Cms growth. Asparagine and methionine were shown to be of major importance. However, combinations of these stimulative compounds mentioned in the literature and of several other carbon and nitrogen sources did not significantly increase the plating efficiency of Cms in pour plates as compared with the non-supplemented basal medium. This may be explained by the use of the absorbance of a bacterial suspension at 605 nm as a parameter for utilization of certain compounds by Cms in the literature, whereas for our objective, plating efficiency was a more useful parameter.

SCM, which was developed for the closely related *C. michiganensis* subsp. *michiganensis* (Fatmi and Schaad, 1988), showed complete inhibition of Cms colonies in the pour platings of this semi-selective medium. Subsequent experiments (Table 2) showed the inhibiting effect of nalidixic acid at 10 mg/l on colonies of Cms, providing a possible explanation for the complete inhibition of Cms on SCM which contained 30 mg/l.

Three inhibitors added to YGM or YPG suppressed colony growth of saprophytes in pour plates at about maximum plating efficiency of Cms. The agar well diffusion technique can be used to determine the critical suppressive concentration of one inhibitor at the edge of the zone of inhibition (Barry, 1980). We modified the agar diffusion test to obtain qualitative data of the optimal concentrations and combinations of inhibitors. The method provided an efficient tool for this purpose. The quantitative determinations of the effect of antibiotics, alone and in combinations, was efficiently determined in 24-well tissue culture plates. The most effective combination of inhibitors added to YGM (YGMI) was: polymyxin B sulphate (an antibiotic that increases cell permeability for most gram-negative bacteria; Pratt and Fekety, 1986) at 30 mg/l, nalidixic acid (synthetic antibiotic inhibiting DNA-replication, in general inhibiting gram-negative bacteria more than gram-positive bacteria; Pratt and Fekety, 1986) at 2 mg/l and S-0208 at 125 mg/l (selectively inhibits gram-negative bacteria, mechanism of action unknown). The average plating efficiency in YGMI compared with that in YGM for Cms strains was c. 60%, for saprophytes from cattle slurry 2 to 3%, and for

saprophytes from potato extract 20%. The much greater inhibiting effect of YGMI on saprophytes from cattle slurry compared with saprophytes from potato heel ends illustrates the differences between bacterial populations in these substrates and the need to develop a selective medium specifically for the type of sample to be tested.

Differentiation between Cms and saprophyte colonies

The long incubation period needed for Cms to form visible colonies in comparison with other bacteria was the main cause for the morphological difference between Cms and saprophyte colonies in the agar. The colonies of the cattle slurry saprophytes in YGMI were in general large and often irregularly shaped, whereas the colonies of Cms were small thin platelets with a slightly bluish appearance.

IFC staining of Cms colonies proved successful for reliable serological differentiation between Cms and colonies of other bacteria in pour plates. Incidentally present yellow-green fluorescent colonies in the non-inoculated cattle slurry could be easily distinguished from the morphologically different and green Cms colonies in the YGMI pour plates. Furthermore, preabsorption of anti-Cms serum with cross-reacting antigen has proven valuable for increasing specificity (Underberg and Sander, 1991). In contrast with *Erwinia* spp. (Van Vuurde and Roozen, 1990), the detection level of Cms by pour plating was greatly affected by colonies of saprophytes. Our experiments showed a strong reduction in the recovery of Cms in the 9 cm diameter YGMI pour plates if more than 100 saprophyte colonies were present (Fig. 1). The detectability of Cms in artificially inoculated cattle slurry was between 10^3 and 10^4 cfu per ml for pour plating in YGMI in 9 cm Petri dishes, and c. 10^5 in 16 mm wells of 24-well tissue culture plates. For *Erwinia chrysanthemi*, growing only slightly slower than most saprophytes in pour plates, 64% recovery was obtained at saprophyte concentration 5,000 per 16 mm diameter well. This resulted in a detection level of 10^2 cfu per ml (Van Vuurde and Roozen, 1990).

The IFC micro colony technique showed that small colonies of Cms can already be detected in the agar after 24 h incubation. The contrast between IF-positive colonies of Cms and background was optimal for pour plates with 1% or less agar, and stained and washed with 0.1% Tween 20 in the buffer.

Comparison of YGMI-pour plating and IFC with IF-cell staining

If good recovery of the target bacterium can be obtained, as was demonstrated for *Erwinia* spp. in cattle slurry (Van Vuurde and Roozen, 1990) IFC has the following advantages over IF: 1) Only viable bacteria form colonies. 2) Larger quantities of sample extract can be investigated with pour plating and IFC than with IF cell-staining. In a standard Petri dish, up to 5 ml of sample extract can be pour plated and up to 100 μl in a 16 mm diameter well of a tissue culture plate. 3) Inspection of IFC preparations can be done faster and is more convenient because of the low objective magnification and the lack of interfering sample particles in the preparation compared with IF-preparations. 4) Colonies which are IFC-positive after the staining procedure still contain viable cells. This allows isolation for further identification of the positive colony. 5) In case IFC-positive colonies of cross-reacting bacteria are found with a Cms-type colony appearance, the selectivity of the medium can be further developed to inhibit this bacterium.

Strategy for Cms detection

For detection of Cms we developed the following strategy, which should be evaluated by large scale testing of cattle slurry samples: 1) Pour plate 10-fold dilutions of the sample with YGMI in 9 cm diameter Petri dishes and incubate at 20 °C for 5 days. 2) Inspect plates containing less than c. 500 colonies under a stereo-microscope with darkfield illumination at c. 30 × total magnification for small, thin, bluish colonies. If these colonies are present, isolate from Cms-type colony by puncturing the colony with a fine needle or glass capillary, followed by streaking on YGM to obtain a pure culture. 4) Identify the pure culture with IF-cell staining, fatty acid profiling, and or eggplant inoculation (Janse and Van Vaerenbergh, 1987; Lelliot and Stead, 1987). If many Cms-type colonies are present and quantification is needed perform IFC on representative areas.

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